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A Modified Elek Test for Detection of Toxigenic Corynebacteria in the Diagnostic Laboratory

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The detection of toxigenicity among *Corynebacterium diphtheriae* and *Corynebacterium ulcerans* strains is the most important test for the microbiological diagnosis of diphtheria. Difficulties with current methods, in particular the Elek test, are well documented. We therefore describe a modified Elek test which provides an accurate result after only 16 h of incubation, in contrast to 48 h for the conventional test.

Diphtheria caused by toxigenic strains of *Corynebacterium diphtheriae* is a disease which has reached epidemic proportions within the European region of the World Health Organization, in particular in eastern Europe, and is causing concern globally (7). Rapid microbiological confirmation of a clinical diagnosis is crucial for epidemiological control purposes and as specific treatment is effective only if administered during the early stages. Currently, the only in vitro method readily available to the majority of diagnostic laboratories is the Elek immunoprecipitation test, first described in 1949, which is both technically demanding and prone to misinterpretation (2). Difficulties with the application of the Elek test have been reported (12): in a United Kingdom national external quality assessment scheme conducted in 1984, only 69% (120 of 173) of participating laboratories obtained the correct result for all four strains of *C. diphtheriae* distributed. Although the clarity and accuracy of the test have been improved following the modification of the Elek medium (1), the misinterpretation of nonspecific precipitin lines remains common, particularly in laboratories where the test is performed infrequently. In national external quality assessment scheme distributions conducted between 1993 and 1995, of the small number of laboratories reporting results for toxigenicity tests (less than 25% of participating laboratories), only 78 and 88% obtained a correct result on the two occasions when nontoxigenic strains were distributed, compared with over 90% when toxigenic strains were distributed (11). PCR has been used for the detection of the diphtheria toxin gene (8–10), in particular the biologically active (A) fragment, and is simple and rapid; however, isolates of *C. diphtheriae* which possess the toxin gene but which do not express a biologically active protein and are therefore for diagnostic purposes nontoxigenic have been found (6, 10). Although such isolates are relatively rare worldwide, PCR alone cannot provide a definitive result; additionally, the necessary technology to perform PCR may not be available to many laboratories, and for these reasons continued phenotypic testing, e.g., the Elek test, is necessary. We therefore describe a modified Elek test based on the methodology used in Russia and Ukraine (5).

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Isolates. Strains of corynebacteria were selected from those referred to the Streptococcus and Diphtheria Reference Unit (SDRU), Central Public Health Laboratory, Colindale, London, United Kingdom, between 1988 and 1995. For both the conventional and modified Elek tests three control strains were used. Two were strains of *C. diphtheriae* subsp. *gravis*, one (NCTC 10648) being a strong toxin producer and the other (NCTC 3984) being a weak toxin producer. The third strain (NCTC 10356) was a nontoxigenic strain of *C. diphtheriae* subsp. *belfanti.*

Elek toxigenicity tests. The Elek base was prepared as described previously (1, 3), and toxigenicity of strains was determined by the conventional and modified Elek tests. For the conventional Elek test (1, 3), newborn bovine serum (NBS) (3 ml) (ICN Biomedicals, Thame, United Kingdom) was added to 15 ml of molten Elek base at 45°C, and 9-cm-diameter plates were poured. Plates were inoculated with the test strain and the three control strains, and an antitoxin strip containing 500 IU/ml was placed on the plate as shown in Fig. 1.

For the modified Elek test, NBS (0.5 ml) was added to 2.5 ml of molten Elek base at 45°C, and 4.5-cm-diameter plates were poured. Plates were inoculated with the test strain and the three control strains, and an antitoxin disc (10 IU/disc) was placed on the plate as shown in Fig. 1.

All plates were incubated for 48 h at 37°C in air and examined for precipitin lines of identity at 16, 24, and 48 h by two individuals (Fig. 2).

Development and optimization of the modified Elek test. The modified Elek test was optimized in terms of thickness (volume) of medium, concentration of antitoxin, and inoculum density and distance from the antitoxin disc. Various volumes (1, 2, 3, 4, 5, 6, 8, and 10 ml/plate) of Elek medium (Elek base supplemented with 16.6% NBS) were added to 4.5-cm-diameter petri dishes and used to determine the effect of agar thickness (volume) on the detection of toxigenicity. Various concentrations (2.5, 5, 7.5, 10, and 12.5 IU/disc) of diphtheria antitoxin (Pasteur Mérieux, Lyon, France) were applied to blank filter discs (6.5-mm diameter; Mast Diagnostics Ltd.). Discs were dried at 37°C for 1 h and used to determine the effect of antitoxin concentration on the detection of toxigenicity. Plates were inoculated at various distances (3, 6, 9, 12, and 15 mm) from the edge of the antitoxin disc (10 IU/disc) with a light, medium, or heavy inoculum to determine the effects of density and distance of inocula on detection of toxigenicity.

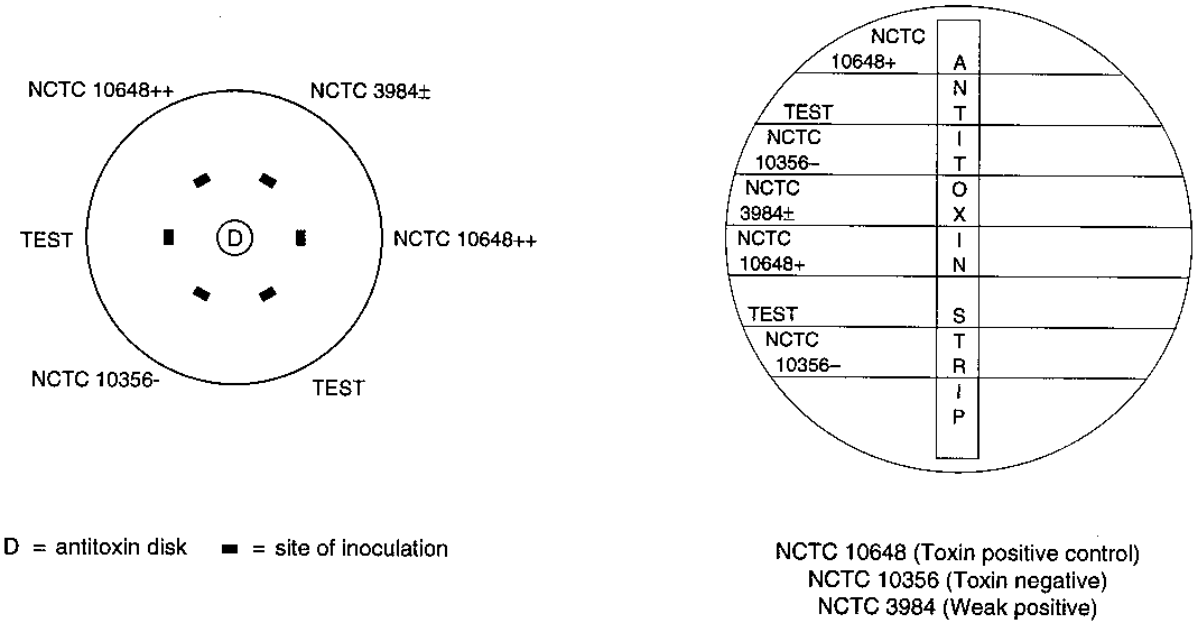

FIG. 1. Templates for the modified (left) and conventional (right) Elek tests.

The thickness (volume) of the medium was found to be a critical factor in increasing the speed and sensitivity of detection of toxigenicity in the modified Elek test, and a reduction in volume improved both parameters. The use of 3 ml of medium (2.5 ml of Elek base and 0.5 ml of NBS) reduced the time taken for the appearance of the immunoprecipitation lines (precipitin lines being clearly visible after 16 h of incubation at 37°C) and furthermore increased the sharpness and visibility of the lines; volumes of less than 3 ml were unsuitable, as they did not provide an adequate covering of the plate.

The concentration of antitoxin had little effect on the speed and sensitivity of the detection of toxigenicity, with similar results being obtained with all concentrations used; discs prepared with 10 IU of antitoxin (20 μl of a 500-IU/ml stock solution) were used for all subsequent experiments.

Both the density and distance of inoculum from the antitoxin disc were found to be important factors in the speed of detection of toxigenicity. Immunoprecipitation lines were clearer and produced more rapidly when a heavy inoculum was used. The optimum distance between the inoculum and the antitoxin disc was found to be 9 mm. At distances of less than 9 mm, the bacterial growth obscured the precipitin lines, whereas at distances of greater than 9 mm, the time taken for the appearance of the lines increased: a minimum of 24 h of incubation was required at a distance of 12 mm, and over 48 h of incubation was required at 15 mm.

The optimum conditions for the modified Elek test were therefore found to be 3 ml of Elek medium (2.5 ml of base and 0.5 ml of NBS) in a 4.5-cm-diameter petri dish, an antitoxin concentration of 10 IU/disc, and a heavy inoculum placed 9

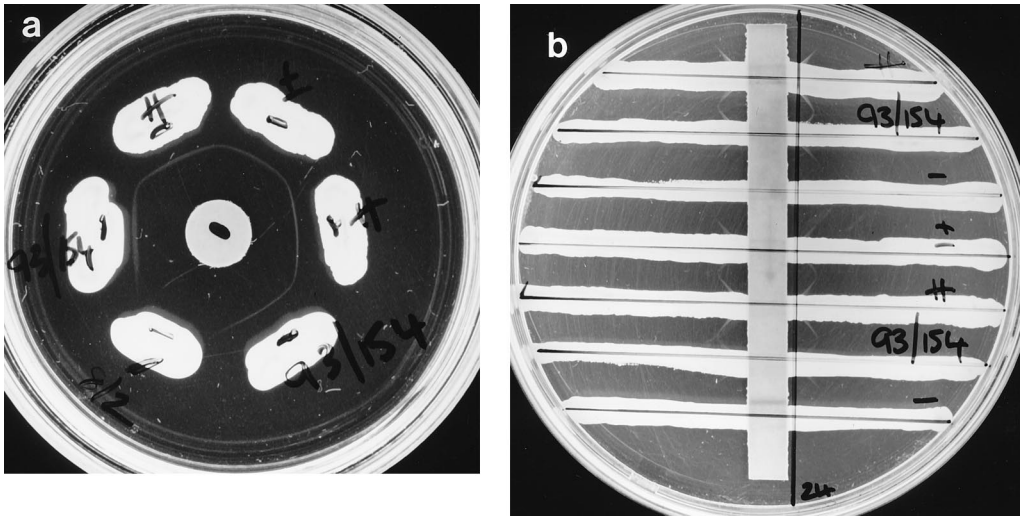

FIG. 2. Detection of a toxigenic test strain with the modified (a) and conventional (b) Elek tests, after 24 h of incubation at 37°C. Plates were inoculated as shown in the templates in Fig. 1.

TABLE 1. Detection of toxigenicity among corynebacteria by the modified and conventional Elek tests

Corynebacterium biotype (no. of isolates)	Toxigenicity[a]	No. of isolates at:					
		16 h by[b]:		24 h by:		48 h by:	
		CE	ME	CE	ME	CE	ME
C. diphtheriae subsp. *gravis* (35)	Tox^+	7	7	7	7	7	7
	Tox^-	28	28	28	28	28	28
C. diphtheriae subsp. *mitis* (37)	Tox^+	16	20	17	21	21	21
	Tox^-	21	17	20	16	16	16
C. diphtheriae subsp. *intermedius* (3)	Tox^+	3	3	3	3	3	3
	Tox^-	0	0	0	0	0	0
C. diphtheriae subsp. *belfanti* (3)	Tox^+	0	0	0	0	0	0
	Tox^-	3	3	3	3	3	3
C. pseudodiphtheriticum (2)	Tox^+	0	0	0	0	0	0
	Tox^-	2	2	2	2	2	2
C. pseudotuberculosis (4)	Tox^+	1	1	1	1	1	1
	Tox^-	3	3	3	3	3	3
C. ulcerans (11)	Tox^+	8	9	9	10	10	10
	Tox^-	3	2	2	1	1	1
Total (95)	Tox^+	35	40	37	42	42	42
	Tox^-	60	55	58	53	53	53
Sensitivity (%) (52 isolates) (95% confidence interval)[c]		85 (62.1–96.8)	90 (68.3–98.8)	90 (68.3–98.8)	100 (83.2–100)	100 (83.2–100)	100 (83.2–100)

[a] Tox^+, toxigenic; Tox^-, nontoxigenic.
[b] CE, conventional Elek test; ME, modified Elek test.
[c] Sensitivity determined by comparison to the subcutaneous virulence test in the guinea pig using 52 of the 95 isolates as follows: *C. diphtheriae* subsp. *gravis*, 1 Tox^+ and 13 Tox^-; *C. diphtheriae* subsp. *mitis*, 16 Tox^+ and 15 Tox^-; *C. diphtheriae* subsp. *belfanti*, 1 Tox^-; *C. diphtheriae* subsp. *intermedius*, 1 Tox^+; *C. ulcerans*, 4 Tox^+ and 1 Tox^-.

mm from the edge of the antitoxin disc. A template (Fig. 1) was used to ensure the accurate positioning of the inoculum and antitoxin disc.

Comparison of the modified and conventional Elek tests. A comparative study of the modified and conventional Elek tests was undertaken by the examination of 95 isolates of corynebacteria referred to the SDRU between 1988 and 1995; the biotypes of the strains examined are given in Table 1. The "gold standard" for the determination of toxin production is the in vivo subcutaneous virulence test in the guinea pig, and the result of this test was known for 52 of the 95 isolates (4); to avoid further in vivo testing, the sensitivity of each test was determined with this smaller group of isolates.

The results of the comparative study of the modified and conventional Elek tests are summarized in Table 1. The conventional test at 48 h and modified test at 24 and 48 h gave identical results for all 95 isolates examined; the agreement between the two tests was 100% (with a 95% confidence interval of 96.0 to 100%). Thus, with the modified test a final result may be obtained and reported after 24 h of incubation as opposed to a total incubation period of 48 h recommended for the conventional test.

The production of nonspecific lines of precipitation after prolonged incubation (e.g., 48 h) is well-known and is a common cause of misinterpretation. Nonspecific lines of precipitation were produced with the modified Elek test (after 48 h of incubation) and were seen as a hexagon 2 mm inside the precipitin lines of identification. However, when reading the optimized modified test at 16 to 24 h, nonspecific lines of precipitation were not visible, thus eliminating some of the problems of misinterpretation inherent in the Elek test. The sensitivities of the two methods were determined by comparison with the in vivo subcutaneous virulence test in the guinea pig. The sensitivity of the conventional test was found to be 100% only after 48 h of incubation. In comparison, with the modified test, 100% sensitivity was obtained after 24 h of incubation and was comparable to the result for the conventional test at 48 h, again indicating that the modified test may be read at 24 h and a final result may then be reported, with a result comparable to that of the conventional test when read at 48 h.

Since January 1996, the modified and conventional tests have been used in parallel for the testing of toxigenicity of all isolates referred to the SDRU (192 isolates); the biotypes and toxigenicities of the strains tested are given in Table 2. No discrepancies between the result of the modified test at 24 h and the conventional test at 48 h have been found.

To conclude, a modified sensitive and specific Elek test which simplifies the toxigenicity testing of corynebacteria for the diagnostic microbiology laboratory has been developed.

TABLE 2. Biotypes and toxigenicity of 192 isolates tested by the modified and conventional Elek tests between January and July 1996

Biotype	Toxigenicity[a]	No. of isolates
C. diphtheriae subsp. *gravis*	Tox^+	7
	Tox^-	107
C. diphtheriae subsp. *mitis*	Tox^+	25
	Tox^-	24
C. diphtheriae subsp. *belfanti*	Tox^-	4
C. ulcerans	Tox^+	5
C. pseudodiphtheriticum	Tox^-	14
Other *Corynebacterium* spp.	Tox^-	6

[a] Tox^+, toxigenic; Tox^-, nontoxigenic.

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REFERENCES

1. **Colman, G., E. Weaver, and A. Efstratiou.** 1992. Screening tests for pathogenic corynebacteria. J. Clin. Pathol. **45:**46–48.
2. **Efstratiou, A., and R. C. George.** 1996. Microbiology and epidemiology of diphtheria. Rev. Med. Microbiol. **7:**31–42.
3. **Efstratiou, A., and P. A. C. Maple.** 1994. Manual for the laboratory diagnosis of diphtheria. ICP/EPI038 (C). Expanded Programme on Immunisation in the European Region of WHO, Copenhagen, Denmark.
4. **Efstratiou, A., K. H. Engler, A. Sangrador, C. Dawes, B. D. Cookson, M. Corbel, and D. Sesardic.** Unpublished data.
5. **Feldman, Y. M., L. G. Makhaneva, and T. G. Glushkevich.** 1987. Determination of the *Corynebacterium diphtheriae* toxigenicity due to indicator paper disks. Russ. J. Microbiol. **4:**32–33.
6. **Groman, N., N. Cianciotto, M. Bjorn, and M. Rabin.** 1983. Detection and expression of DNA homologous to the *tox* gene in nontoxigenic isolates of *Corynebacterium diphtheriae*. Infect. Immun. **42:**48–56.
7. **Hardy, I. R. B., S. Dittman, and R. W. Sutter.** 1996. Current situation and control strategies for resurgence of diphtheria in newly independent states of the former Soviet Union. Lancet **347:**1739–1744.
8. **Hauser, D., M. R. Popoff, M. Kiredjian, P. Boquet, and F. Bimet.** 1993. Polymerase chain reaction assay for diagnosis of potentially toxinogenic *Corynebacterium diphtheriae* strains: correlation with ADP-ribosylation activity assay. J. Clin. Microbiol. **31:**2720–2723.
9. **Mikhailovich, V. M., M. G. Melnikov, I. K. Mazurova, I. K. Wachsmuth, J. D. Wenger, M. Wharton, H. Nakao, and T. Popovic.** 1995. Application of PCR for detection of toxigenic *Corynebacterium diphtheriae* strains isolated during the Russian diphtheria epidemic, 1990 through 1994. J. Clin. Microbiol. **33:**3061–3063.
10. **Pallen, M. J., A. J. Hay, L. H. Puckey, and A. Efstratiou.** 1994. Polymerase chain reaction for screening clinical isolates of corynebacteria for the production of diphtheria toxin. J. Clin. Pathol. **47:**353–356.
11. **Snell, J. J. S.** 1996. Personal communication.
12. **Snell, J. J. S., J. V. de Mello, P. S. Gardner, W. Kwantes, and R. Brooks.** 1984. Detection of toxin production by *Corynebacterium diphtheriae*: the results of a trial organised as part of the United Kingdom Scheme. J. Clin. Pathol. **37:**796–799.